Thermostable Gluc amylase

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a division of application number 09/821,616, filed March 29, 2001, which is a continuation of application no. 09/199,290 filed November 24, 1998, which is a continuation-in-part of application nos. 08/979,673 and 09/107,657 filed November 26, 1997 and June 30, 1998, respectively, and claims priority under 35 U.S.C. 119 of Danish application nos. 1557/97 and PA 1998 00925 filed December 30, 1997 and July 10, 1998, respectively, and U.S. application nos. 60/070,746 and 60/094,344 filed January 8, 1998 and July 28, 1998, respectively, the contents of which are fully incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to a thermostable glucoamylase suitable for, *e.g.*, starch conversion, *e.g.*, for producing glucose from starch. The present invention also relates to the use of said thermostable glucoamylase in various processes, in particular in the saccharification step in starch convention processes.

BACKGROUND OF THE INVENTION

Glucoamylases (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) are enzymes which catalyze the release of D-glucose from the non-reducing ends of starch or related oligo- and polysaccharide molecules.

Glucoamylases are produced by several filamentous fungi and yeasts, including *Aspergillus niger* and *Aspergillus awamori.*

Commercially, the glucoamylases are used to convert corn starch which is already partially hydrolyzed by an α-amylase to glucose. The glucose may further be converted by glucose isomerase to a mixture composed almost equally of glucose and fructose. This mixture, or the mixture further enriched with fructose, is the commonly used high fructose corn syrup commercialized throughout the world. This syrup is the world's largest tonnage product produced by an enzymatic process. The three enzymes involved in the conversion of starch to fructose are among the most important industrial enzymes produced.

One of the main problems existing with regard to the commercial use of glucoamylase in the production of high fructose corn syrup is the relatively low thermal stability of glucoamylases, such as the commercially available *Aspergillus niger* glucoamylase (*i.e.*, (sold as AMG by Novo Nordisk A/S). The commercial *Aspergillus* glucoamylase is not as thermally stable as α-amylase or

glucose isomerase and it is most active and stable at lower pH's than either α-amylase or glucose isomerase. Accordingly, it must be used in a separate vessel at a lower temperature and pH.

US patent no. 4,247,637 describes a thermostable glucoamylase having a molecular weight of about 31,000 Da derived from *Talaromyces duponti* suitable for saccharifying a liquefied starch solution to a syrup. The glucoamylase is stated to retain at least about 90% of its initial glucoamylase activity when held at 70°C for 10 minutes at pH 4.5.

US patent no. 4,587,215 discloses a thermostable amyloglucosidase derived from the species *Talaromyces thermophilus* with a molecular weight of about 45,000 Da. The disclosed amyloglucosidase (or glucoamylase) loses its enzymatic activity in two distinct phases, an initial period of rapid decay followed by a period of slow decay. At 70°C (pH=5.0) the half-life for the fast decay is about 18 minutes with no measurable loss of activity within an hour in the second phase of decay.

Bunni L et al., (1989), Enzyme Microb. Technol., Vol. 11, p. 370-375. concerns production, isolation and partial characterization of an extracellular amylolytic system composed of at least one form of α-amylase and one form of an α-glucosidase produced by *Talaromyces emersonii* CBS 814.70. Only the α-amylase is isolated, purified and characterized.

BRIEF DISCLOSURE OF THE INVENTION

The present invention is based upon the finding of a novel thermostable glucoamylase suitable for use, *e.g.,* in the saccharification step in starch conversion processes.

The terms "glucoamylase" and "AMG" are used interchangeably below.

The thermal stability of the glucoamylase of the invention is measured as $T_{1/2}$ (half-life) using the method described in the "Materials and Methods" section below.

The inventors of the present invention have isolated, purified and characterized a thermostable glucoamylase from a strain of *Talaromyces emersonii* now deposited with the Centraalbureau voor Schimmelcultures under the number CBS 793.97.

When applied to a protein, the term "isolated" indicates that the protein is found in a condition other than its native environment. In a preferred form, the isolated protein is substantially free of other proteins, particularly other homologous proteins (*i.e.,* "homologous impurities" (see below)).

It is preferred to provide the protein in a greater than 40% pure form, more preferably greater than 60% pure form. Even more preferably it is preferred to provide the protein in a highly purified form, *i.e.,* greater than 80% pure, more preferably greater than 95% pure, and even more preferably greater than 99% pure, as determined by SDS-PAGE.

The term "isolated enzyme" may alternatively be termed "purified enzyme".

The term "homologous impurities" means any impurity (*e.g.* another polypeptide than the polypeptide of the invention) which originates from the homologous cell, from where the polypeptide of the invention is originally obtained.

The isolated glucoamylase has a very high thermal stability in comparison to prior art glucoamylases, such as the *Aspergillus niger* glucoamylase (available from Novo Nordisk A/S under the trade name AMG). The T½ (half-life) was determined to be about 120 minutes at 70°C (pH 4.5) as described in Example 2 below. The T½ of the recombinant *T. emersonii* AMG expressed in yeast was determined to be about 110 minutes as described in Example 12.

Therefore, in the first aspect the present invention relates to an isolated enzyme with glucoamylase activity having a $T_{1/2}$ (half-life) of at least 100 minutes in 50 mM NaOAc, 0.2 AGU/ml, pH 4.5, at 70°C.

In the second aspect the invention relates to an enzyme with glucoamylase activity comprising one or more of the partial sequences shown in SEQ ID Nos. 1-6 or the full length enzyme shown in SEQ ID NO: 7 or an enzyme with glucoamylase activity being substantially homologous thereto.

The term "partial sequence" denotes a partial polypeptide sequence which is comprised in a longer polypeptide sequence, wherein said longer polypeptide sequence is having the activity of interest.

The invention also relates to the cloned DNA sequence encoding the glucoamylase of the invention.

Further, the invention also relates to a process of converting starch or partially hydrolyzed starch into a syrup containing, *e.g.,* dextrose, said process including the step of saccharifying starch hydrolyzate in the presence of a glucoamylase of the invention.

It is an object of the invention to provide a method of saccharifying a liquefied starch solution, wherein an enzymatic saccharification is carried out using a glucoamylase of the invention.

Furthermore, the invention relates to the use of a glucoamylase of the invention in a starch conversion process, such as a continuous starch conversion process. In an embodiment of the continuous starch conversion process it includes a continuous saccharification step.

The glucoamylase of the invention may also be used in processes for producing oligosaccharides or specialty syrups.

Finally, the invention relates to an isolated pure culture of the microorganism *Talaromyces emersonii* CBS 793.97 or a mutant thereof capable of producing a glucoamylase of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the SDS-PAGE gel (stained with Coomassie Blue) used for determining the molecular weight (M_w) of the purified *Talaromyces emersonii* CBS 793.97 glucoamylase of the present invention.

1: Standard marker,

2: Q Sepharose pool (1. run)

3: S Sepharose pool;

Figure 2 shows the pH activity profile of *Talaromyces emersonii* and *Aspergillus niger* glucoamylase (AMG) in 0.5% maltose at 60°C;

Figure 3 shows the temperature activity profile of the *Talaromyces emersonii* CBS 793.97 glucoamylase vs. *Aspergillus niger* glucoamylase (AMG)

Figure 4 shows the curve for determining $T_{1/2}$ (half-life) in 50 mM NaOAc, 0.2 AGU/ml, pH 4.5, at 70°C of *Talaromyces emersonii* CBS 793.97 glucoamylase vs. *Aspergillus niger* glucoamylase (AMG);

Figure 5 shows the sequence of the *Talaromyces emersonii* AMG locus. The predicted amino acid sequence is shown below the nucleotide sequence. The four introns are shown in lower case letters. Consensus introns sequences are underlined. Putative signal and pro-peptides are double underlined and dotted underline, respectively;

Figure 6 shows an alignment/comparison of the amino acid sequences of the *A. niger* AMG (An_amg-1.pro), *A. oryzae* AMG Ao_AMG.pro), and *Talaromyces emersonii* AMG (Tal-AMG.pro). Identical amino acid residues are indicated by a *.

Signal and pro peptides are underlined by a single and a double lined, respectively;

Figure 7 shows the *Aspergillus* expression cassette pCaHj483 used in Example 5;

Figure 8 shows the *Aspergillus* expression plasmid, pJal518, for the *Talaromyces emersonii* AMG gene;

Figure 9 shows the construction of *A. niger* disruption plasmid;

Figure 10 shows the SDS page gel of two transformants, JaL228#5.77 and HowB112#8.10, expressing the *Talaromyces emersonii* glucoamylase of the invention. JaL228 and HowB112 are the untransformed parent strains. MW: Promega's Protein Molecular;

Figure 11 shows the thermal stability of the *T. emersonii* AMG produced the strain *A. niger* HowB112 determined in 50mM NaOAC, pH 4.5, 70°C, 0.2 AGU/ml (T1/2 determined to 20 minutes);

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Figure 12 compares the thermal stability at 68°C of the fermentation broth of *T. emersonii* AMG expressed in yeast produced in yeast and the *A. niger* AMG;

Figure 13 shows the result of the test for determining the thermostability of recombinant *Talaromyces emersonii* AMG produced in yeast at 70°C, pH 4.5, 0.2 AGU/ml. T1/2 was determined to about 110°C.

DETAILED DISCLOSURE OF THE INVENTION

The present invention is based upon the finding of a novel thermostable glucoamylase suitable for use in, *e.g.,* the saccharification step in a starch conversion process.

The inventors of the present invention have isolated, purified and characterized a glucoamylase from a strain of *Talaromyces emersonii* CBS 793.97. The glucoamylase turned out to have a very high thermal stability in comparison to prior art glucoamylases.

Accordingly, in a first aspect the present invention relates to an isolated enzyme with glucoamylase activity having a $T_{1/2}$ (half-life) of at least 100 minutes, such as between 100 and 140 minutes, in 50 mM NaOAc, 0.2 AGU/ml, pH 4.5, at 70°C.

T1/2 (half-life) of the isolated *Talaromyces emersonii* CBS 793.97 glucoamylase was determined to be about 120 minutes at 70°C as described in Example 2 below and to be about 110°C for the *T. emersonii* produced in yeast as described in Example 12.

The molecular weight of the isolated glucoamylase was found to be about 70 kDa determined by SDS-PAGE. Further, the pI of said enzyme was determined to be below 3.5 using isoelectrical focusing.

The isoelectric point, pI, is defined as the pH value where the enzyme molecule complex (with optionally attached metal or other ions) is neutral, *i.e.,* the sum of electrostatic charges (net electrostatic charge, NEC) on the complex is equal to zero. In this sum of course consideration of the positive or negative nature of the electrostatic charge must be taken into account.

It is expected that substantially homologous enzymes having the same advantageous properties are obtainable from other micro-organisms, especially fungal organisms such as filamentous fungi, in particular from another strain of *Talaromyces*, especially another strains of *Talaromyces emersonii*.

The deposited micro-organism

An isolate of the filamentous fungus strain, from which the glucoamylase of the invention has been isolated, has been deposited with the Centraalbureau voor Schimmelcultures, P.O. Box 273, 3740 AG Baarn, the Netherlands, for the purposes of patent procedure on the date indicated

below. CBS being an international depository under the Budapest Treaty affords permanence of the deposit in accordance with rule 9 of said treaty.

Deposit date : June 2, 1997

Depositor's ref.: NN049253

CBS designation : CBS 793.97

The isolate of the filamentous fungus *Talaromyces emersonii* CBS No. 793.97 has been deposited under conditions that assure that access to the isolated fungus will be available during the pendency of this patent application to one determined by the commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. § 1.14 and 35 U.S.C § 122. The deposit represents a substantially pure culture of the isolated fungus. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Talaromyces emersonii glucoamylase amino acid sequence

The inventors have sequenced the thermostable glucoamylase derived from *Talaromyces emersonii* CBS 793.97 as will be described further in the Example 3 below. According to the invention the *Talaromyces* AMG may have a Asp145Asn (or D145N) substitution (using SEQ ID NO: 7 numbering).

Therefore, the invention also relates to an isolated enzyme with glucoamylase activity comprising one or more of the partial sequences shown in SEQ ID NOS: 1-6 or the full length sequence shown in SEQ ID NO: 7 or an enzyme with glucoamylase activity being substantially homologous thereto. SEQ ID NO: 34 shows the full length sequence including the signal and pre propeptide from amino acid no. 1 to 27.

Homology of the protein sequence

The homology between two glucoamylases is determined as the degree of identity between the two protein sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as gap provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular

Biology, 48, p. 443-453). Using gap with the following settings for polypeptide sequence comparison: gap creation penalty of 3.0 and gap extension penalty of 0.1.

According to the invention a "substantially homologous" amino acid sequence exhibits a degree of identity preferably of at least 80%, at least 90%, more preferably at least 95%, more preferably at least 97%, and most preferably at least 99% with the partial amino acid sequences shown in SEQ ID NO: 1-6 or SEQ ID NO: 7.

The Cloned *Talaromyces emersonii* DNA sequence

The invention also relates to a cloned DNA sequence encoding an enzyme exhibiting glucoamylase activity of the invention, which DNA sequence comprises:

(a) the glucoamylase encoding part of the DNA sequence shown in SEQ ID NO: 33;

(b) the DNA sequence shown in positions 649-2724 in SEQ ID NO:33 or its complementary strand;

(c) an analogue of the DNA sequence defined in (a) or (b) which is at least 80% homologous with said DNA sequence;

(d) a DNA sequence which hybridizes with a double-stranded DNA probe comprising the sequence shown in 649-2724 in SEQ ID NO: 33 at low stringency;

(e) a DNA sequence which, because of the degeneracy of the genetic code, does not hybridize with the sequences of (b) or (f), but which codes for a polypeptide having exactly the same amino acid sequence as the polypeptide encoded by any of these DNA sequences; or

(g) a DNA sequence which is a fragment of the DNA sequences specified in (a), (b), (c), (d), or (e).

The mature part of the AMG of the invention is encoded by the DNA sequence in position 728-2724 of SEQ ID NO: 33. When expressing the AMG of the invention in yeast, e.g., *Saccharomyces cerevisiae* YNG318, the introns need to be cut out as described in Example 7.

Homology of DNA sequences

The DNA sequence homology referred to above is determined as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711)(Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation

penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the analogous DNA sequences referred to above exhibits a degree of identity preferably of at least 80%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97% with the AMG encoding part of the DNA sequence shown in SEQ ID NO: 33 or the glucoamylase encoding part with or witout introns.

<u>Hybridization:</u>

The hybridization conditions referred to above to define an analogous DNA sequence as defined in d) above which hybridizes to a double-stranded DNA probe comprising the sequence shown in positions 649-2748 in SEQ ID NO: 33 (*i.e.*, the AMG encoding part), under at least low stringency conditions, but preferably at medium or high stringency conditions are as described in detail below.

Suitable experimental conditions for determining hybridization at low, medium, or high stringency between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al. 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at about 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at about 55°C (low stringency), more preferably at about 60°C (medium stringency), still more preferably at about 65°C (medium/high stringency), even more preferably at about 70°C (high stringency), and even more preferably at about 75°C (very high stringency).

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using a x-ray film.

<u>Starch conversion</u>

The present invention provides a method of using the thermostable glucoamylase of the invention for producing glucose and the like from starch. Generally, the method includes the steps of partially hydrolyzing precursor starch in the presence of α-amylase and then further hydrolyzing the release of D-glucose from the non-reducing ends of the starch or related oligo- and polysaccharide molecules in the presence of glucoamylase by cleaving α-(1⊠4) and α-(1⊠6) glucosidic bonds.

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The partial hydrolysis of the precursor starch utilizing α-amylase provides an initial breakdown of the starch molecules by hydrolyzing internal α-(1⊠4)-linkages. In commercial applications, the initial hydrolysis using α-amylase is run at a temperature of approximately 105°C. A very high starch concentration is processed, usually 30% to 40% solids. The initial hydrolysis is usually carried out for five minutes at this elevated temperature. The partially hydrolyzed starch can then be transferred to a second tank and incubated for approximately one hour at a temperature of 85° to 90°C to derive a dextrose equivalent (D.E.) of 10 to 15.

The step of further hydrolyzing the release of D-glucose from the non-reducing ends of the starch or related oligo- and polysaccharides molecules in the presence of glucoamylase is normally carried out in a separate tank at a reduced temperature between 30° and 60°C. Preferably the temperature of the substrate liquid is dropped to between 55° and 60°C. The pH of the solution is dropped from 6 to 6.5 to a range between 3 and 5.5. Preferably, the pH of the solution is 4 to 4.5. The glucoamylase is added to the solution and the reaction is carried out for 24-72 hours, preferably 36-48 hours.

By using a thermostable glucoamylase of the invention saccharification processes may be carried out at a higher temperature than traditional batch saccharification processes. According to the invention saccharification may be carried out at temperatures in the range from above 60-80°C, preferably 63-75°C. This applies both for traditional batch processes (described above) and for continuous saccharification processes.

Actually, continuous saccharification processes including one or more membrane separation steps, i.e., filtration steps, must be carried out at temperatures of above 60°C to be able to maintain a reasonably high flux over the membrane. Therefore, a thermostable glucoamylase of the invention provides the possibility of carrying out large scale continuous saccharification processes at a fair price within and period of time acceptable for industrial saccharification processes. According to the invention the saccharification time may even be shortened.

The activity of a glucoamylase of the invention is generally substantially higher at temperatures between 60°C-80°C than at the traditionally used temperature between 30-60°C. Therefore, by increasing the temperature at which the glucoamylase operates the saccharification process may be carried out within a shorter period of time or the process may be carried out using lower enzyme dosage.

As the thermal stability of the glucoamylase of the invention is very high in comparison to, e.g., the commercially available *Aspergillus niger* glucoamylase (*i.e.*, AMG) a less amount of glucoamylase needs to be added to replace the glucoamylase being inactivated during the saccharification process. More glucoamylase is maintained active during saccharification process

according to the present invention. Furthermore, the risk of microbial contamination is also reduced when carrying the saccharification process at temperature above 63°C.

By using a glucoamylase with increased specific activity (measured as activity towards maltose), a lower enzyme dosage may be required in the saccharification process.

Examples of saccharification processes, wherein the glucoamylase of the invention may advantageously be used include the processes described in JP 3-224493; JP 1-191693; JP 62-272987; and EP 452,238.

In a further aspect the invention relates to a method of saccharifying a liquefied starch solution, which method comprises an enzymatic saccharification step using a glucoamylase of the invention.

The glucoamylase of the invention may be used in the present inventive process in combination with an enzyme that hydrolyzes only α-(1⊠6)-glucosidic bonds in molecules with at least four glucosyl residues. Preferably, the glucoamylase of the invention is used in combination with pullulanase or isoamylase. The use of isoamylase and pullulanase for debranching, the molecular properties of the enzymes, and the potential use of the enzymes with glucoamylase is set forth in G.M.A. van Beynum et al., Starch Conversion Technology, Marcel Dekker, New York, 1985, 101-142.

In a further aspect the invention relates to the use of a glucoamylase of the invention in a starch conversion process.

Further, the glucoamylase of the invention may be used in a continuous starch conversion process including a continuous saccharification step.

The glucoamylase of the invention may also be used in immobilised form. This is suitable and often used for producing speciality syrups, such as maltose syrups, and further for the raffinate stream of oligosaccharides in connection with the production of fructose syrups.

The glucoamylase of the invention may also be used in a process for producing ethanol for fuel or beverage or may be used in a fermentation process for producing organic compounds, such as citric acid, ascorbic acid, lysine, glutamic acid.

MATERIALS AND METHODS
Material
Enzymes:

Glucoamylase derived from the deposited filamentous fungus *Talaromyces emersonii* CBS No. 793.97 hasbeen deposited with the Centraalbureau voor Schimmelcultures, P.O. Box 273, 3740 AG Baarn, the Netherlands, for the purposes of patent procedure on the date indicated

below. CBS being an international depository under the Budapest Treaty affords permanence of the deposit in accordance with rule 9 of said treaty.

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Depositor's ref.: NN049253

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Glucoamylase G1 derived from *Aspergillus niger* disclosed in Boel et al. (1984), EMBO J. 3 (5), 1097-1102, available from Novo Nordisk and shown in SEQ ID NO: 9.

Strains:

JaL228; Construction of this strain is described in WO98/12300

SMO110; Construction of this strain is described in Example 6

Yeast Strain: *Saccharomyces cerevisiae* YNG318: MATa leu2-D2 ura3-52 his4-539 pep4-D1[cir+].

Genes:

A. niger G1 glucoamylase gene is shown in SEQ ID NO: 8

T. emersonii glucoamylase gene with introns is shown in fig. 5 and SEQ ID NO: 33. The introns are shown in Fig. 5.

Plasmids:

pJSO026 (*S. cerevisiae* expression plasmid)(J.S.Okkels, (1996)"A URA3-promoter deletion in a pYES vector increases the expression level of a fungal lipase in *Saccharomyces cerevisiae*. Recombinant DNA Biotechnology III: The Integration of Biological and Engineering Sciences, vol. 782 of the Annals of the New York Academy of Sciences) More specifically, the expression plasmid pJSO26, is derived from pYES 2.0 by replacing the inducible GAL1-promoter of pYES 2.0 with the constitutively expressed TPI (triose phosphate isomerase)-promoter from *Saccharomyces cerevisiae* (Albert and Karwasaki, (1982), J. Mol. Appl Genet., 1, 419-434), and deleting a part of the URA3 promoter.

pJaL497; Construction of this plasmid is described in Example 5

pJaL507; Construction of this plasmid is described in Example 5

pJaL510; Construction of this plasmid is described in Example 5

pJaL511; Construction of this plasmid is described in Example 5

pJaL518; Construction of this plasmid is described in Example 6

pCaHj483; Construction of this plasmid is described in Example 6

pJRoy10; Construction of this plasmid is described in Example 6

pJRoy17; Construction of this plasmid is described in Example 6

pSMO127; Construction of this plasmid is described in Example 6

pCR™II; Available from Invitrogen Corporation, San Diego, CA, USA.

Equipment:

Automatic DNA Sequencer (Applied Biosystems Model 377)

Media:

SC-ura medium:

Yeast Nitrogen w/o ami   7.5 g

Bernsteinsaüre (Ravsyre)   11.3 g

NaOH   6.8 g

Casaminoacid w/o vit   5.6 g

Tryptophan   0.1 g

Dest. water ad   1000 ml

Autoclaved for 20 minutes at 121°C.

From a sterile stock solution of 5% Threonin 4 ml is added to a volume of 900 ml together with 100 ml of a sterile 20% glucose.

YPD medium:

Yeast extract   10 g

Peptone   20 g

Dest. water ad   1000 ml

Autoclaved for 20 minutes at 121°C

100 ml of a sterile 20% glucose is added to 900 ml.

Methods:

Determination of AGU activity

One Novo Amyloglucosidase Unit (AGU) is defined as the amount of enzyme which hydrolyzes 1 micromole maltose per minute under the following standard conditions:

Substrate. . . . . . maltose

Temperature. . . . . 25°C

pH. . . . . .4.3 (acetate buffer)

Reaction time. . . . 30 minutes

A detailed description of the analytical method (AF22) is available on request.

Determination of PUN activity

PUN is defined as the amount of enzyme which hydrolyzes pullulan (0.2 % pullulan, 40°C, pH 5.0), liberating reducing carbohydrate with a reducing power equivalent to 1 micro-mol glucose pr. minute.

Determination of AFAU activity

The activity is determined in AFAU calculated as the reduction in starch concentration at pH 2.5, 40°C, 0.17 g/l starch and determined by an iodine-starch reaction.

Thermal Stability I (T½ (half-life) determination of AMG

The thermal stability of glucoamylase (determined as T½ (half-life)) is tested using the following method: 950 microliter 50 mM sodium acetate buffer (pH 4.5) (NaOAc) is incubated for 5 minutes at 70°C. 50 microliter enzyme in buffer (4 AGU/ml) is added. 2 x 40 microliter samples are taken at fixed periods between 0 and 360 minutes and chilled on ice. After chilling the samples the residual enzyme activity is measured using the AGU determination assay (described above). The activity (AGU/ml) measured before incubation (0 minutes) is used as reference (100%). $T_{1/2}$ is the period of time until which the percent relative activity is decreased to 50%.

Determination of thermal stability II

1600 microliter of a supernatant and 400 microliter of 0.5 M NaAC pH 4.5 is mixed.

7 eppendorph tubes each containing 250 microliter of the mixture are incubated in a Perkin Elmer thermocycler at 68°C or 70°C for 0, 5, 10, 20, 30, 45 and 60 minutes.

100 microliter from each mixture is mixed with 100 microliter of 5 mM CNPG3 (2-chloro-4-Nitrophenyl-Alpha-Maltotrioside from genzyme) in microtiterwells. After incubation for 30 minutes at 37°C the absorbance is measured at 405 nm.

Determination of Specific Activity of a glucoamylase

750 microL substrate is incubated 5 minutes at selected temperatures,such as 37°C, 60°C or 70°C.

50 microL enzyme diluted in sodium acetate is added, and the activity was determined using the AGU standard method described above. The kinetic parameters: Kcat and Km are

measured at 45°C by adding 50 microL enzyme diluted in sodium acetate to preheated 750 microL substrate. Aliquots of 100 microL are removed after 0, 3, 6, 9 and 12 minutes and transferred to 100 microL 0.4 M Sodium hydroxide to stop the reaction. A blank is included.

20 microL is transferred to a Micro titre plates and 200 microL GOD-Perid solution is added. Absorbance is measured at 650 nm after 30 minutes incubation at room temperature.

Glucose is used as standard, and the specific activity is calculated as k_{cat} (sec.$^{-1}$)

Transformation of Aspergillus oryzae (general procedure)

100 ml of YPD (Sherman et al., (1981), Methods in Yeast Genetics, Cold Spring Harbor Laboratory) is inoculated with spores of A. oryzae and incubated with shaking for about 24 hours. The mycelium is harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium is suspended in 15 ml of 1.2 M $MgSO_4$, 10 mM NaH_2PO_4, pH 5.8. The suspension is cooled on ice and 1 ml of buffer containing 120 mg of Novozym^{TM} 234 is added. After 5 min., 1 ml of 12 mg/ml BSA (Sigma type H25) is added and incubation with gentle agitation continued for 1.5-2.5 hours at 37C until a large number of protoplasts is visible in a sample inspected under the microscope.

The suspension is filtered through miracloth, the filtrate transferred to a sterile tube and overlayed with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH 7.0. Centrifugation is performed for 15 min. at 1000 g and the protoplasts are collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) are added to the protoplast suspension and the mixture is centrifugated for 5 min. at 1000 g. The protoplast pellet is resuspended in 3 ml of STC and repelleted. This is repeated. Finally, the protoplasts are resuspended in 0.2-1 ml of STC.

100 µl of protoplast suspension are mixed with 5-25 µg of p3SR2 (an A. nidulans amdS gene carrying plasmid described in Hynes et al., Mol. and Cel. Biol., Vol. 3, No. 8, 1430-1439, Aug. 1983) in 10 µl of STC. The mixture is left at room temperature for 25 min. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution are added and carefully mixed. The mixture is left at room temperature for 25 min., spun at 2.500 g for 15 min. and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, (1966), Biochem. Biophys. Acta 113, 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37C spores are picked, suspended in sterile water and spread

for single colonies. This procedure is repeated and spores of a single colony after the second re-isolation are stored as a defined transformant.

Fed batch fermentation

Fed batch fermentation is performed in a medium comprising maltodextrin as a carbon source, urea as a nitrogen source and yeast extract. The fed batch fermentation is performed by inoculating a shake flask culture of fungal host cells in question into a medium comprising 3.5% of the carbon source and 0.5% of the nitrogen source. After 24 hours of cultivation at pH 5.0 and 34°C the continuous supply of additional carbon and nitrogen sources are initiated. The carbon source is kept as the limiting factor and it is secured that oxygen is present in excess. The fed batch cultivation is continued for 4 days, after which the enzymes can be recovered by centrifugation, ultrafiltration, clear filtration and germ filtration. Further purification may be done by anionexchange chromatographic methods known in the art.

Transformation of *Saccharomyces cerevisiae* YNG318

The DNA fragments and the opened vectors are mixed and transformed into the yeast *Saccharomyces cerevisiae* YNG318 by standard methods.

EXAMPLES

Example 1

Purification

3500 ml *T. emersonii* culture broth from wild-type fermentation with 0.05 AGU/ml was centrifuged at 9000 rpm followed by vacuum filtration through filter paper and finally a blank filtration. The following procedure was then used to purify the enzyme:

Phenyl Sepharose (250 ml): 1.3 M AMS/10 mM Tris/2 mM $CaCl_2$, pH 7; elution with 10 mM Tris/2 mM $CaCl_2$, pH 7.

Dialysis: 20 mM NaAc, 2 mM $CaCl_2$, pH 5.

Q Sepharose (100 ml): 20 mM NaAc, 2mM $CaCl_2$, pH 5; elution with a linear gradient from 0-0.4 M NaCl over 10 column volumes.

Dialysis: 20 mM NaAc, 2 mM $CaCl_2$, pH 5.

Colour removal: 0.5% coal in 10 minutes.

Q Sepharose (20 ml): 20 mM NaAc, 2 mM $CaCl_2$, pH 4.5; elution with a linear gradient from 0-0.4 M NaCl over 10 column volumes.

Dialysis: 20 mM NaAc, 2mM $CaCl_2$, pH 5.

S Sepharose (1 ml):   5 mM citric acid, pH 2.9; elution with a linear gradient from 0-0.3 M NaCl over 10 column volume.

A purity of the enzyme of more than 90% was obtained after the S Sepharose step.

Example 2

Characterisation of the _Talaromyces emersonii_ glucoamylase

The purified _Talaromyces emersonii_ CBS 793.97 glucoamylase was used for characterisation.

Molecular weight (M_w)

The molecular weight was determined by SDS-PAGE to around 70 kDa as shown in Figure 1.

pI

The pI was determined to lie below 3.5 by isoelectrical focusing (Amploline PAG, pH 3.5-9.5 from Pharmacia).

pH profile

The pH-activity dependency of the _Talaromyces emersonii_ glucoamylase was determined and compared with profile of _Aspergillus niger_ glucoamylase.

The pH activity profile was determined using 0.5% maltose as substrate in 0.1 M sodium acetate at 60°C. The pH was measured in duple samples comprising 0.1-1 AGU/ml. The result of the test is shown in Figure 2.

Temperature profile

The temperature-activity dependency of the _Talaromyces emersonii_ glucoamylase of the invention was determined and compared with the profile of _Aspergillus niger_ glucoamylase.

200 µl 0.5% maltose, pH 4.3 was incubated at 37, 50, 60, 70, 75, 80 and 90°C and the reaction was started by adding 10 µl enzyme (0.25 AGU/ml); reaction time was 10 minutes. The result of the test is shown in Figure 3.

Temperature stability - T½ (half-life)

The thermal stability of the _Talaromyces emersonii_ glucoamylase was determined and compared with the thermal stability of _Aspergillus niger_ glucoamylase.

The method used is described above in the "Material and Methods" section as "Thermal Stability I (T½ (half-life) determination of AMG".

The T½ of the *Talaromyces emersonii* glucoamylase was determined to about 120 minutes at 70°C. The T½ of the *Aspergillus niger* glucoamylase was determined to 7 minutes under the same conditions (See Figure 4).

Specific activity

The extension coefficient was determined to: ε = 2.44 ml/mg*cm on basis of absorbency at 280 nm and protein concentration. The specific activity towards maltose at 37°C was then calculated to 7.3 AGU/mg. Purity of the sample was approximately 90% and a corrected specific activity is therefore 8.0 AGU/mg. Following specific activities were measured:

AMG	Specific activity (AGU/mg)		
	37°C	60°C	70°C
T. emersonii	8.0	21	27
A. niger	2.0	6.6	8.0

*) Estimated for pure enzyme.

EXAMPLE 3

Sequencing of the N-terminal of *T. emersonii* glucoamylase

The N-terminal amino acid sequence of *T. emersonii* glucoamylase was determined following SDS-PAGE and electroblotting onto a PVDF-membrane. Peptides were derived from reduced and S-carboxymethylated glucoamylase by cleaving with a lysyl-specific protease. The resulting peptides were fractionated and re-purified using RP-HPLC before subjected to N-terminal sequence determination.

N-terminal sequence (SEQ ID NO: 1):

Ala Asn Gly Ser Leu Asp Ser Phe Leu Ala Thr Glu Xaa Pro Ile Ala Leu Gln Gly Val Leu Asn Asn Ile Gly

Peptide 1 (SEQ ID NO: 2):

Val Gln Thr Ile Ser Asn Pro Ser Gly Asp Leu Ser Thr Gly Gly Leu Gly Glu Pro Lys

Peptide 2 (SEQ ID NO: 3):

Xaa Asn Val Asn Glu Thr Ala Phe Thr Gly Pro Xaa Gly Arg Pro Gln Arg Asp Gly Pro Ala Leu

Peptide 3 (SEQ ID NO: 4):

Asp Val Asn Ser Ile Leu Gly Ser Ile His Thr Phe Asp Pro Ala Gly Gly Cys Asp Asp Ser Thr Phe
Gln Pro Cys Ser Ala Arg Ala Leu Ala Asn His Lys

Peptide 4 (SEQ ID NO: 5):

Thr Xaa Ala Ala Ala Glu Gln Leu Tyr Asp Ala Ile Tyr Gln Trp Lys

Peptide 5 (SEQ ID NO: 6):

Ala Gln Thr Asp Gly Thr Ile Val Trp Glu Asp Asp Pro Asn Arg Ser Tyr Thr Val Pro Ala Tyr Cys
Gly Gln Thr Thr Ala Ile Leu Asp Asp Ser Trp Gln
Xaa denoted a residue that could not be assigned.

EXAMPLE 4

The full length *T. emersonii* glucoamylase

The full length *T. emersonii* glucoamylase amino acid sequence shown in SEQ ID NO: 7 was identified using standard methods.

Example 5

Cloning and sequencing of the *Talaromyces emersonii* glucoamylase gene

PCR cloning parts of the *Talaromyces emersonii* AMG gene

For cloning of the *Talaromyces emersonii* AMG gene degenerated primers shown in table 1 was designed for PCR amplification of part of the AMG gene.

Table 1

Primer no:	Sequence	Comments
102434(SEQ ID NO:10) 102435(SEQ ID NO:11)	V L N N I G 5'-GTNTTRAAYAAYATHGG 5'-GTNCTNAAYAAYATHGG	N-Terminal 5' primers
117360(SEQ ID NO:12) 117361(SEQ ID NO:13)	D L W E E V CTRGANACCCTYCTYCA-5' CTRAAYACCCTYCTYCA-5'	Active site consensus 3' primers
	W E D D P N	C-Terminal

| 127420(SEQ ID NO:14) | ACCCTYCTRCTRGGNTT-5' | 3' primers |

Genomic DNA *from Talaromyces emersonii* was prepared from protoplasts made by standard procedures [cf.e.g., Christensen et al. Biotechnology 1989 6 1419-1422] and was used as template in the PCR reaction. Amplification reaction were performed in 100 µl volumes containing 2.5 units Taq-polymerase, 100 ng of *A. oryzae* genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH 8.0,1.5 mM MgCl$_2$, 250 nM of each dNTP, and 100pM of each of the following primers sets: 102434/117360, 102434/117361, 102435/117360, 102434/117361, 102434/127420, and 102434/127420.

Amplification was carried out in a Perkin-Elmer Cetus DNA Termal 480, and consisted of one cycle of 3 minutes at 94°C, followed by 30 cycles of 1 minutes at 94°C, 30 seconds at 40°C, and 1 minutes at 72°C. Only the PCR reaction 102434/117360 gave products. Four bands was detected with the following sizes 1400, 800, 650, and 525bp. All four bands were purified and cloned into the vector pCR®2.1 (Invitrogen®). Sequencing of a few clone from each band and sequence comparisons to the *A. niger* AMG, releaved that a clone from the 650 bp band encodes for the N-terminal part of the Talaromyces emersonii AMG. This clone was designated pJaL497.

To obtained more of the gene a specific primer (123036: 5'-GTGAGCCCAAGTTCAATGTG- 3' (SEQ ID NO:15) was made out from the sequence of clone pJaL497. The primer set 123036/127420 was used for PCR on Talaromyces genomic DNA and a single fragment on 1500 bp was obtained. The PCR fragment was clone into the vector pCR®2.1 and sequenced. By sequencing the clone was confirmed to encoded the C-terminal part of the *Talaromyces emersonii* AMG. The clone was designated pJaL507.

Genomic restriction mapping and cloning of a genomic clone(s)

Taken together the two clones pJaL497 and pJaL507 covered about 95% of the AMG gene. In order to clone the missing part of the AMG gene a genomic restriction map was constructed by using the two PCR fragment as probes to a Southern blot of Talaromyces emersonii genomic DNA digested with single or a combination of a number of restriction enzymes. This shows that the Talaromyces emersonii AMG gene is located on two EcoRI fragment on about 5.6 kb and 6.3 kb, respectively.

Talaromyces emersonii genomic DNA was digested with EcoRI and fragments with the size between 4-7 kb was purified and used for construction of a partially genome library in Lambda ZAP II as described by the manufactory instruction(Stratagene). The library was first

screened using the 0.7 kb EcoRI fragment from pJaL497 (encoding the N-terminal half of the AMG gene) as probe to get the start of the AMG gene. One clone was obtained and designated pJaL511. In a second screening of the library using a 0.75 kb EcoRV fragment from pJaL507 (encoding the C-terminal half of the AMG gene) as probe in order to get the C-terminal end of the AMG gene. One clone was obtained and designated pJaL510.

Sequence analysis of the _Talaromyces emersonii_ AMG gene

The AMG gene sequence was obtained by sequencing on the plasmids: pJaL497, pJaL507, pJaL510, and pJaL511 and on subclones hereof with the standard reverse and forward primers for pUC. Remaining gabs were closed by using specific oligonucleotide as primers.

Potential introns were found by comparing the sequence with consensus sequences for introns in _Aspergillus_ and with the _A. niger_ AMG sequence. The _Talaromyces emersonii_ AMG nucleotide sequence has an open reading frame encoding a protein on 618 amino acid, interrupted by four introns of 57 bp, 55 bp, 48 bp, and 59 bp, respectively. The nucleotide sequence (with introns) and deduced amino acid sequence is shown in Fig. 5. The DNA sequence (with introns) is also shown in SEQ ID NO: 33 and the _Talaromyces emersonii_ AMG sequence (with signal sequence from 1 to 27) is shown in SEQ ID NO: 34. Comparison of the deduced amino acid sequence with the _A. oryzae_ AMG and _A. niger_ AMG shows an identity of 60.1 % and 60.5 %, respectively. Alignment of the amino acid sequences shown in Fig. 6 shows that the _Talaromyces_ AMG has a very short hinge between the catalytic domain and the starch binding domain, which is also seen for the _A. oryzae_ AMG.

Example 6

Construction of the _Aspergillus_ vector pCaHj483

Construction of pCaHj483 is depicted in Fig. 7. Said plasmid is build from the following fragments:

a) The vector pToC65 (WO 91/17243) cut with _EcoRI_ and _XbaI_.

b) A 2.7 kb _XbaI_ fragment from _A. nidulans_ carrying the amdS gene (C. M. Corrick et al., Gene 53, (1987), 63-71). The amdS gene is used as a selective marker in fungal transformations. The amdS gene has been modified so that the _BamHI_ site normally present in the gene is destroyed. This has been done by introducing a silent point mutation using the primer:

5'-AGAAATCGGGTATCCTTTCAG- 3'   (SEQ ID NO:16)

c) A 0.6 kb *EcoRI/BamHI* fragment carrying the *A. niger* NA2 promoter fused to a 60bp DNA fragment of the sequence encoding the 5´ untranslated end of the mRNA of the *A. nidulans tpi* gene. The NA2 promoter was isolated from the plasmid pNA2 (described in WO 89/01969) and fused to the 60 bp *tpi* sequence by PCR. The primer encoding the 60 bp *tpi* sequence had the following sequence:

5'-GCTCCTCATGGTGGATCCCCAGTTGTGTATATAGAGGATTGAGGAAGGAAGAGAA GTGTGGATAGAGGTAAATTGAGTTGGAAACTCCAAGCATGGCATCCTTGC - 3´ (SEQ ID NO:17)

d) A 675 bp *XbaI* fragment carrying the *A. niger* glucoamylase transcription terminator. The fragment was isolated from the plasmid pICAMG/Term (described in EP 0238 023).

The *BamHI* site of fragment c was connected to the *XbaI* site in front of the transcription terminator on fragment d via the pIC19R linker (*BamHI* to *XbaI*)

Construction of a AMG expression plasmid, pJaL518

The coding region of the *Talaromyces emersonii* AMG gene was amplified by PCR, using the following two oligonucleotides primers: 139746:

5'-GACAGATCTCC<u>ACCATGGCGTCCCTCGTTG</u> 3' (SEQ ID NO:18); and

primer 139747:

5'-GACCTCGAG<u>TCACTGCCAACTATCGTC</u> 3' (SEQ ID NO:19). The underlined regions indicate sequences present in the *Talaromyces emersonii* AMG gene. To facilitate cloning a restriction enzyme site was inserted into the 5' end of each primer; primer 139746 contains a BglII site and primer 139747 contains a XhoI site.

Talaromyces emersonii genomic DNA was used as template in the PCR reaction. The reaction was performed in a volume of 100 µl containing 2.5 units Taq polymerase, 100 ng of pSO2, 250 nM of each dNTP, and 10 pmol of each of the two primers described above in a reaction buffer of 50 mM KCl, 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$.

Amplification was carried out in a Perkin-Elmer Cetus DNA Termal 480, and consisted of one cycle of 3 minutes at 94°C, followed by 25 cycles of 1 minute at 94°C, 30 seconds at 55°C, and 1 minute at 72°C. The PCR reaction produced a single DNA fragment of 2099 bp in length. This fragment was digested with BglII and XhoI and isolated by gel electrophoresis, purified, and cloned into pCaHj483 digested with BamHI and XhoI, resulting in a plasmid which was designated pJaL518. Thus, the construction of the plasmid pJal518 resulted in a fungal expression plasmid for the *Talaromyces emersonii* AMG gene (Fig. 8).

<u>Construction of the *Aspergillus niger* strain, SMO110</u>

1. Cloning of *A. niger* pyrG gene

A library of *A. niger* BO-1 was created in EMBL4 as described by the manufactory instructions. The library was screened with a DIG labelled oligonucleotides (PyrG: 5'-CCCTCACCAGGGGAATGCTGCAGTTGATG- 3' (SEQ ID NO:20) which was designed from the published *Aspergillus niger* sequence (Wilson et al. Nucleic Acids Res. 16, (1988), 2339-2339). A positive EMBL4 clone which hybridized to the DIG probe was isolated from the BO-1 library, and a 3.9 kb Xba1 fragment containing the pyrG gene was subcloned from the EMBL4 clone and clone into pUC118 to create pJRoy10.

2. Cloning of the *A. niger* glucoamylase (AMG) gene

The above *A. niger* BO-1 library was screened with a DIG labelled PCR fragment generated by amplification on *A. niger* genomic DNA with the following oligonucleotides, 950847:

5'-CGCCATTCTCGGCGACTT-3' (SEQ ID NO:21),and

oligonucleotide 951216:

5'-CGCCGCGGTATTCTGCAG-3' (SEQ ID NO:22), which was designed from the published *Aspergillus niger* sequence (Boel et al., EMBO J. 3, (1984), 1581-1585). A positive EMBL4 clone which hybridized to the DIG probe was isolated from the BO-1 library, and a 4.0 kb SpeI fragment containing the AMG gene was subcloned from the EMBL4 clone and clone into pBluescriptSK+ generating plasmid pJRoy17a.

3. Construction of the *A. niger* AMG Disruption Cassette

A 2.3 kb SpeI-XhoI fragment containing *pyrG* was gel isolated from pJRoy10 and the restricted ends filled in with Klenow polymerase. The fragment was inserted into the BglII site of pJRoy17 which cuts within the AMG gene creating plasmid pSMO127 (Fig. 9). Between the two SpeI sites of pSMO127a is contained the 2.3 kb *pyrG* gene flanked by 2.2 kb and 2.3 kb 5' and 3' AMG, respectively.

4. Construction of a *A. niger* strain disrupted for AMG, SMO110

A. niger JRoyP3 is a spontaneously pyrG mutant of *A. niger* BO-1, which was selected for the growth on a plate containing 5'-fluoro-orotic acid (5'-FOA). The pyrG gene encodes orotidine 5'-phosphate carboxylase and its deficient mutant can be characterized as uridine

auxotroph. The identity of pyrG mutant was confirmed by the complementation of the growth on a minimal medium with *A. nidulans* pyrG gene.

Twenty micrograms of the plasmid pSMO127 was digested with SpeI. The DNA was resolved on an 0.8% agarose gel and the 6 kb consisting of the linear disruption cassette was gel isolated. The linear DNA was transformed into strain JRoyP3.

Genomic DNA was prepared from 200 transformants which was then digested with SpeI. The gel-resolved DNA was transferred to a hybond nylon filter, and hybridized to a non-radioactive DIG probe consisting of the AMG open reading frame. A gene replacement of the disruption cassette into the AMG locus would result in an increase of the wild type 4 kb AMG band to 6.3 kb, an increase due to the 2.3 kb *pyrG* gene. One transformant #110 with the above characteristics was selected for further analysis.

The transformant #110 were grown in 25 ml shake flasks containing YPM media. Strains BO-1 and parent strain JRoyP3 were grown as AMG producing controls. After 3 days, 30µl of clear supernatants were run on a 8-16% SDS PAGE Novex gel. No AMG band was seen in transformant #110, while large bands of AMG were produced in the positive control strain BO-1 and parent strain JRoyP3. Transformant #110 was named SMO110.

Expression of *Talaromyces emersonii* AMG in *Aspergillus oryzae* and *Aspergillus niger*

The strains JaL228 and SMO110 was transformed with pJaL518 as described by Christensen et al.; Biotechnology 1988 6 1419-1422. Typically, *A. oryzae* mycelia was grown in a rich nutrient broth. The mycelia were separated from the broth by filtration. The enzyme preparation Novozyme® (Novo Nordisk) was added to the mycelia in osmotically stabilizing buffer such as 1.2 M $MgSO_4$ buffered to pH 5.0 with sodium phosphate. The suspension was incubated for 60 minutes at 37°C with agitation. The protoplast was filtered through mira-cloth to remove mycelial debris. The protoplast was harvested and washed twice with STC (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5). The protoplast was finally resuspended in 200-1000 µl STC.

For transformation 5 µg DNA was added to 100 µl protoplast suspension and then 200 µl PEG solution (60% PEG 4000, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5) was added and the mixture was incubated for 20 minutes at room temperature. The protoplast were harvested and washed twice with 1.2 M sorbitol. The protoplast was finally resuspended 200 µl 1.2 M sorbitol, plated on selective plates (minimal medium + 10 g/l Bacto-Agar (Difco), and incubated at 37°C. After 3-4 days of growth at 37°C, stable transformants appear as vigorously growing and sporulating colonies. Transformants was spore isolated twice.

Transformants was grown in shake flask for 4 days at 30°C in 100 ml YPM medium (2 g/l yeast extract, 2 g/l peptone, and 2% maltose). Supernatants were tested for AMG activity as described and analyzed on SDS page gel (Fig. 10).

EXAMPLE 7

Removal of the four introns from the *Talaromyces emersonii* AMG DNA sequence for expression in yeast.

For each exon a PCR reaction was made with primers containing overlap to the next exon. Tal 1 and Tal 4 contain an overlap with the yeast vector pJSO026.

Exon 1: Tal 1 was used as the 5' primer and Tal 5 as the 3' primer and the genomic sequence coding for AMG was used as the template. Exon 2: Tal 6 was used as the 5' primer and Tal 7 was used as the 3' primer and the genomic sequence coding for AMG was used as the template. Exon 3: Tal 8 was used as the 5' primer and Tal 9 was used as the 3' primer and the genomic sequence coding for AMG was used as the template. Exon 4: Tal 10 was used as the 5' primer and Tal 11 was used as the 3' primer and the genomic sequence coding for AMG was used as the template. Exon 5: Tal 12 was used as the 5' primer and Tal 4 was used as the 3' primer and the genomic sequence coding for AMG was used as the template.

A final PCR reaction was performed to combine the 5 exons to a sequence containing the complete coding sequence. In this PCR reaction the 5 fragments from the first PCR reactions were used as template and Tal 1 was used as the 5' primer and Tal4 was used as the 3' primer.

This final PCR fragment containing the coding region was used in an in vivo recombination in yeast together with pJSO026 cut with the restriction enzymes Smal(or BamHI) and Xbal (to remove the coding region and at the same time create an overlap of about 20 bp in each end to make a recombination event possible).

Tal 1: 5'-CAA TAT AAA CGA CGG TAC CCG GGA GAT CTC CAC CATG GCG TCC CTC GTT G-3' (SEQ ID NO:23);

Tal 4: 5'-CTA ATT ACA TCA TGC GGC CCT CTA GAT CAC TGC CAA CTA TCG TC-3' (SEQ ID NO:24);

Tal 5: 5'-AAT TTG GGT CGC TCC TGC TCG-3' (SEQ ID NO:25);

Tal 6: 5'-CGA GCA GGA GCG ACC CAA ATT ATT TCT ACT CCT GGA CAC G-3' (SEQ ID NO: 26);

Tal 7: 5'-GAT GAG ATA GTT CGC ATA CG-3' (SEQ ID NO: 27);

Tal 8: 5'-CGT ATG CGA ACT ATC TCA TCG ACA ACG GCG AGG CTT CGA CTG C-3' (SEQ ID NO:28);

Tal 9: 5'-CGA AGG TGG ATG AGT TCC AG-3' (SEQ ID NO: 29);

Tal 10: 5'-CTG GAA CTC ATC CAC CTT CGA CCT CTG GGA AGA AGT AGA AGG-3' (SEQ ID NO: 30)

Tal 11: 5'-GAC AAT ACT CAG ATA TCC ATC-3' (SEQ ID NO: 31)

Tal 12: 5'-GAT GGA TAT CTG AGT ATT GTC GAG AAA TAT ACT CCC TCA GAC G-3' (SEQ ID NO: 32)

EXAMPLE 8

Expression of *Talaromyces emersonii* glucoamylase in yeast

To express *Talaromyces emersonii* AMG in the yeast *Saccharomyces cerevisiae* YNG318 the yeast expression vector pJSO26 was constructed as described in the "Material and Methods" section above.

PJSO26 comprising the DNA sequence encoding the *Talaromyces* AMG was transformed into the yeast by standard methods (cf. Sambrooks et al., (1989), Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor).

The yeast cells were grown at 30°C for 3 days in Sc-ura medium followed by growth for 3 days in YPD. The culture was then centrifuged and the supernatant was used for the thermostability assay described in the "Materials and Method" section.

Thermal stability of the *Talaromyces* AMG expressed in yeast at 68°C.

The fermentation broth of the *Talaromyces emersonii* AMG expressed in yeast (*Saccharomyces cerevisiae* YNG318) was used for determination of the thermal stability at 68°C using the method described above under "Determination of thermal stability II". The result of the test is shown in Figure 12.

EXAMPLE 9

Purification of recombinant *Talaromyces* AMG produced using *A. niger* HowB112

200 ml culture broth from fermentation of *A. niger* HowB112 harboring the Talaromyces emersonii gene was centrifuged at 9000 rpm and dialyzed against 20 mM NaOac, pH 5 over night. The solution was then applied on a S Sepharose column (200 ml) previously equilibrated in 20 mM NaOAc, pH 5. The glucoamylase was collected in the effluent, and applied on a Q Sepharose column (50 ml) previously equilibrated in 20 mM NaOAC, pH 4.5. Unbound material

was washed of the column and the glucoamylase was eluted using a linear gradient from 0-0.3 M NaCl in 20 mM NaOAc over 10 column volumes. Purity of the glucoamylase fraction was checked by SDS-PAGE and only one single band was seen. The molecular weight was again found to about 70 kdal as seen for the wild type glucoamylase. The specific activity towards maltose was measured and a specific activity of 8.0 AGU/mg (37°C) and 21.0 AGU/mg (60°C) were found which is in accordance the data on the wild type enzyme.

EXAMPLE 10

Kinetic Parameters

Kinetic Parameters for Hydrolysis of Maltose and Isomaltose by *Aspergillus niger* AMG and the recombinant *Talaromyces emersonii* AMG expressed in *A. niger*.

Maltose	k_{cat} (s^{-1})[a]	K_m (mM)	k_{cat}/K_m (s^{-1}mM^{-1})
Talaromyces emersonii	30.6	3.8	8.1
Aspergillus niger	10.7	1.2	8.8

[a] At 45°C uusing 0.05 M NaOAc, pH 4.5

Isomaltose	k_{cat} (s^{-1})[a]	K_m (mM)	k_{cat}/K_m (s^{-1}mM^{-1})
Talaromyces emersonii	2.70	53.6	0.050
Aspergillus niger	0.41	19.8	0.021

[a] At 45°C uusing 0.05 M NaOAc, pH 4.5

EXAMPLE 11

Saccharification performance of recombinant *Talaromyces emersonii* AMG produced in *A. niger*

The saccharification performance of the *Talaromyces emersonii* glucoamylase was tested at different temperatures with and without the addition of acid α-amylase and pullulanase. Saccharification was run under the following conditions:

Substrate: 10 DE Maltodextrin, approx. 30% DS (w/w)

Temperatures:60, 65, or 70°C

Initial pH: 4.5

Enzyme dosage:

Recombinant *Talaromyces emersonii* glucoamylase produced *in A. niger*: 0.24 or 0.32 AGU/g DS

Acid α-amylase derived from *A. niger*: 0.020 AFAU/g DS

Pullulanase derived from *Bacillus*:   0.03 PUN/g DS

When used alone *Talaromyces* AMG was dosed at the high dosage (0.32 AGU/g DS), otherwise at the low dosage, i.e., 0.24 AGU/g DS.

Saccharification

The substrate for saccharificationg was made by dissolving maltodextrin (prepared from common corn) in boiling Milli-Q water and adjusting the dry substance to approximately 30% (w/w). pH was adjusted to 4.5 (measured at 60°C). Aliquots of substrate corresponding to 150g dry solids were transferred to 500 ml blue cap glass flasks and placed in a water bath with stirring at the respective temperatures. Enzymes were added and pH readjusted if necessary (measured at incubation temperature). Samples were taken periodically and analysed at HPLC for determination of the carbohydrate composition.

The glucose produced during saccharification are given in the table below, the first three columns representing the saccharification with glucoamylase and acid α-amylase and pullulanase, the last three with glucoamylase alone. Numbers are % DP1 on DS.

| Time | 0.24 AGU+0.02AFAU+0.03PUN | | | 0.32 AGU | | |
(hours)	60°C	65°C	70°C	60°C	65°C	70°C
24	88.96	90.51	87.91	84.98	86.28	84.35
48	94.03	94.28	91.90	88.86	89.51	86.98
72	95.08	94.75	93.12	90.18	90.42	87.99
98	95.03	94.59	93.64	90.65	90.72	88.51

A glucose yield above 95% was obtained after 72 hours using an enzyme dosage of 0.24 AGU/g DS which is corresponding to 0.03 mg/g DS. The typical dosage of A. niger AMG would be 0.18 AGU/g DS which is corresponding to 0.09 mg/g DS to get a yield og 95-96% glucose. A significantly lower enzyme dosage on mg enzyme protein of *Talaromyces* AMG is therefore required in the saccharification process compared to *A. niger* AMG due to the high specific activity of *T. emersonii* AMG.

Example 12

Temperature stability - T½ (half-life) of recombinant *Talaromyces emersonii* AMG expressed in yeast

The thermal stability of recombinant *Talaromyces emersonii* glucoamylase expressed in yeast (purified using the method described in Example 9) was determined at 70°C, pH 4.5, 0.2

AGU/ml using the method described above in the "Material and Methods" section as "Thermal Stability I (T½ (half-life) determination of AMG".

Figure 13 shows the result of the test. The T½ of the recombinant *Talaromyces emersonii* glucoamylase expressed in yeast was determined to about 110 minutes at 70°C.